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# Disruption of a Guard Cell–Expressed Protein Phosphatase 2A Regulatory Subunit, *RCN1*, Confers Abscisic Acid Insensitivity in Arabidopsis

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**Pharmacological studies have led to a model in which the phytohormone abscisic acid (ABA) may be positively transduced via protein phosphatases of the type 1 (PP1) or type 2A (PP2A) families. However, pharmacological evidence also exists that PP1s or PP2As may function as negative regulators of ABA signaling. Furthermore, recessive disruption mutants in protein phosphatases that function in ABA signal transduction have not yet been identified. A guard cell–expressed PP2A gene, *RCN1*, which had been characterized previously as a molecular component affecting auxin transport and gravity response, was isolated. A T-DNA disruption mutation in *RCN1* confers recessive ABA insensitivity to Arabidopsis. The *rcn1* mutation impairs ABA-induced stomatal closing and ABA activation of slow anion channels. Calcium imaging analyses show a reduced sensitivity of ABA-induced cytosolic calcium increases in *rcn1*, whereas mechanisms downstream of cytosolic calcium increases show wild-type responses, suggesting that RCN1 functions in ABA signal transduction upstream of cytosolic $Ca^{2+}$ increases. Furthermore, *rcn1* shows ABA insensitivity in ABA inhibition of seed germination and ABA-induced gene expression. The PP1 and PP2A inhibitor okadaic acid phenocopies the *rcn1* phenotype in wild-type plants both in ABA-induced cytosolic calcium increases and in seed germination, and the wild-type *RCN1* genomic DNA complements *rcn1* phenotypes. These data show that RCN1 functions as a general positive transducer of early ABA signaling.**

## INTRODUCTION

The phytohormone abscisic acid (ABA) plays important roles in plant growth, development, and cellular signaling (Finkelstein and Zeevaart, 1994; Grill and Himmelbach, 1998; Koornneef et al., 1998; Finkelstein et al., 2002). ABA maintains seed dormancy, controls seed maturation, regulates vegetative growth, and mediates plant responses to various environmental stimuli such as stomatal closure during drought (Grill and Himmelbach, 1998; Koornneef et al., 1998; MacRobbie, 1998; Schroeder et al., 2001; Finkelstein et al., 2002). Previous molecular genetic approaches have led to the identification of several genes that function in ABA signal transduction (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994; Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998; Sheen, 1998; Finkelstein and Lynch, 2000; Li et al., 2000; Hugouvieux et al., 2001; Xiong et al., 2001b).

Guard cells in the leaf epidermis form stomates and regulate $CO_2$ uptake into leaves for photosynthesis and control transpirational water loss. Guard cells integrate water status, hormonal stimuli, light, and other environmental conditions to regulate stomatal apertures for optimization of plant growth and have become a well-developed system in which to characterize events in early plant signaling cascades (for reviews, see MacRobbie, 1998; Schroeder et al., 2001).

Pharmacological studies have suggested that type 1 or 2A protein phosphatases (PP1 or PP2A) act as both negative and positive regulators of ABA signal transduction (Schmidt et al., 1995; Esser et al., 1997; Grabov et al., 1997; Hey et al., 1997; Pei et al., 1997; Wu et al., 1997). Studies showed that okadaic acid (OA), which inhibits PP1s or PP2As, promotes anion channel activation and ABA-induced stomatal closing in *Vicia* and *Commelina* (Schmidt et al.,

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1995). Replacement of cytosolic ATP with nonhydrolyzable analogs or depletion of cytosolic ATP disrupted ABA responses in guard cells, but only in the absence of OA. These data further implicate PP1- or PP2A-induced dephosphorylation events as negative regulators in ABA signaling (Schmidt et al., 1995). Consistent with this model, the PP1/PP2A inhibitor OA stimulated expression of the ABA-induced *RD29A* and *KIN2* genes in tomato hypocotyl cells in the absence of ABA (Wu et al., 1997).

Conversely, studies in other systems showed that OA partially inhibited ABA induction of the *PHAV1* gene in barley aleurone cells (Kuo et al., 1996), and in Arabidopsis guard cells, OA partially inhibited ABA activation of anion channels and stomatal closing (Pei et al., 1997). Together, these studies indicate that both positively transducing PP1s or PP2As (Kuo et al., 1996; Hey et al., 1997; Pei et al., 1997) and negatively regulating PP1s or PP2As (Schmidt et al., 1995; Esser et al., 1997; Grabov et al., 1997; Wu et al., 1997) might function at different locations in ABA signal transduction.

Further pharmacological evidence for conditionally active positively and negatively regulating protein phosphatases was obtained in pea guard cells: OA either inhibits or enhances ABA signaling depending on the physiological status or the history of stomates (Hey et al., 1997). When pea stomates are preopened, OA enhances ABA-induced stomatal closing (Hey et al., 1997), as in *Vicia* and *Commelina* (Schmidt et al., 1995; Esser et al., 1997). But the same study in pea showed that when stomates are closed initially, OA counteracts guard cell ABA responses and abolishes the ABA induction of dehydrin mRNA (Hey et al., 1997). In spite of several independent studies that implicate PP1s or PP2As pharmacologically in ABA signal transduction, genes that encode the proposed PP1 or PP2A protein phosphatases, as well as their locations within ABA signal cascades and their downstream targets, remain unknown.

In addition to PP1/PP2A-mediated ABA signal transduction, other phosphorylation/dephosphorylation enzymes have been shown to mediate ABA signaling in guard cells (for reviews, see Himmelbach et al., 1998; Leung and Giraudat, 1998; Assmann and Shimazaki, 1999). Previous studies have shown roles for type 2C protein phosphatases (PP2Cs), ABA-activated/ABA-responsive protein kinases (AAPK/ABRK), and $Ca^{2+}$-dependent protein kinases in ABA signaling (Leung et al., 1994, 1997; Meyer et al., 1994; Li and Assmann, 1996; Sheen, 1996, 1998; Mori and Muto, 1997; Rodriguez et al., 1998; Li et al., 2000). Dominant mutants in the AAPK kinase disrupt the ABA activation of anion channels and ABA-induced stomatal closure (Li et al., 2000). The dominant mutations *abi1-1* and *abi2-1* impaired ABA-induced anion channel activation and ABA-induced stomatal closing (Pei et al., 1997) and have been shown to disrupt ABA signaling upstream of $Ca^{2+}$ channel activation (Allen et al., 1999a; Murata et al., 2001). However, to date, no recessive gene deletion mutants in kinase and PP2C genes have been characterized that affect ABA signal transduction; therefore, neomorphic effects of the dominant mutants cannot be excluded unequivocally, although several lines of evidence support roles for the wild-type enzymes in ABA signaling (Li and Assmann, 1996; Leung et al., 1997; Mori and Muto, 1997; Sheen, 1998; Li et al., 2000; Merlot et al., 2001; for reviews, see Leung and Giraudat, 1998; Finkelstein et al., 2002).

Here, we identify a guard cell–expressed PP2A regulatory A subunit for which recessive gene disruption shows ABA insensitivity in Arabidopsis and that affects early events in guard cell ABA signal transduction. Further analyses show that this PP2A is required for ABA signal transduction during seed germination and ABA-induced gene expression, suggesting that the RCN1 is a general positive regulator of ABA signal transduction in Arabidopsis.

## RESULTS

### ABA-Insensitive Stomatal Response by Disruption of a Guard Cell–Expressed PP2A Gene

To identify guard cell–expressed PP2A genes, catalytic and regulatory subunit sequences of PP2As were aligned, and then degenerate oligomers designed from conserved regions were used to amplify guard cell–expressed PP2A genes using enriched Arabidopsis guard cell cDNA libraries (see Methods).

One of the genes encoding a PP2A that we identified from guard cell cDNA libraries was *RCN1*. RCN1, a PP2A regulatory A subunit, was characterized previously as a molecular component affecting auxin transport and gravitropism (Garbers et al., 1996; Rashotte et al., 2001). Loss-of-function *rcn1* mutant seedlings, in which no full-length *RCN1* transcript and no protein product were observed (Garbers et al., 1996; Deruère et al., 1999), showed defects in root and hypocotyl elongation and reduced PP2A activity, biochemically demonstrating that RCN1 is an activator of PP2A activity (Garbers et al., 1996; Deruère et al., 1999). To further test the expression of *RCN1* in guard cells, we performed reverse transcription (RT) PCR with total RNA that was prepared independently from highly purified guard cells (>98% pure) and mesophyll cells (>96% pure). As shown in Figure 1A, *RCN1* was expressed in both mesophyll cells and guard cells. The identities of *RCN1* PCR products were confirmed by diagnostic restriction enzyme digestions (data not shown). Densitometry analysis of *RCN1* expression levels suggested a higher expression in guard cells compared with mesophyll cells. Wild-type transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the *RCN1* promoter (3 kb of the *RCN1* upstream sequence; Deruère et al., 1999) also showed that *RCN1* is expressed in guard cells (Figure 1B).

Pharmacological studies have suggested that both positively transducing and negatively regulating PP1s or PP2As may function in ABA signal transduction (see Introduction).

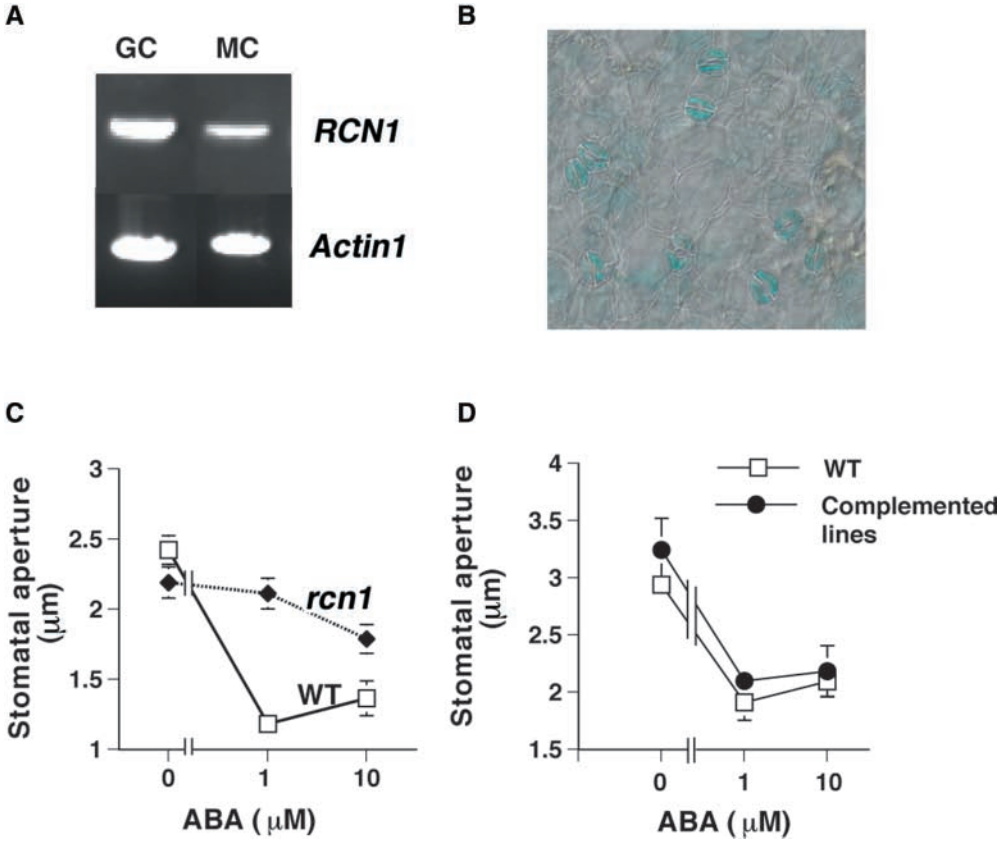


**Figure 1.** RT-PCR and GUS Activity Analyses of *RCN1* in Guard Cells and ABA-Insensitive Stomatal Responses in the *rcn1* Mutant.

**(A)** RT-PCR with RNA extracted from highly purified guard cell (GC) and mesophyll cell (MC) protoplasts. The *Actin1* gene was amplified as a control.

**(B)** The *RCN1* promoter drives GUS activity in guard cells of wild-type plants expressing the RCN1-GUS fusion construct.

**(C)** Stomatal aperture measurements show that ABA-induced stomatal closing is reduced in the *rcn1* T-DNA disruption mutant. Stomatal apertures were measured 3 h after the addition of 1 or 10 μM ABA. Error bars represent standard errors relative to three independent experiments with 36 stomata per data point.

**(D)** ABA-induced stomatal closing was observed in both wild-type and two independent *rcn1*-complemented lines (*rcn1*g17-1 and *rcn1*g8-1). Error bars represent standard errors of two independent experiments with 40 stomata per data point.

Error bars are smaller than symbols when not visible. Note that the light fluence rate was 83 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in **(C)** and 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in **(D)**. WT, wild type.

To determine whether an *rcn1* disruption mutant carrying a T-DNA insertion (Garbers et al., 1996) has enhancing or inhibitory effects on the stomatal response to ABA, we first performed ABA-induced stomatal closing assays. Figure 1C shows that stomatal closing in *rcn1* mutant plants was less sensitive to ABA than that in wild-type plants (Wassilewskija ecotype) at 1 and 10 μM ABA ($P < 0.001$). For complementation analysis, we used *rcn1* plants transformed with a 7-kb genomic fragment containing the *RCN1* gene under the control of its own promoter (1.6 kb of the *RCN1* upstream sequence) (Garbers et al., 1996) in the hygromycin-resistant vector pCIT20 (Ma et al., 1992). The root-curling assay and PCR analyses in T2 generation seedlings showed cosegregation of the rescued phenotype with the *RCN1* transgene (Garbers et al., 1996). Genomic DNA gel blot analyses with a hygromycin resistance marker DNA probe revealed a single T-DNA insertion carrying the complementing construct (J.M. Kwak, unpublished data). In addition, RT-PCR analyses showed that *RCN1* expression was restored in the complemented lines (J.M. Kwak, unpublished data). Two independent T4 homozygous plant lines showed no difference in ABA sensitivity in stomatal aperture measurements compared with wild-type plants, showing complementation of the *rcn1* phenotype ($P > 0.58$ at 1 μM ABA and $P > 0.41$ at 10 μM ABA; Figure 1D). The PP2A inhibitor OA inhibits ABA-induced stomatal closing in Arabidopsis (Pei et al., 1997),

and the *rcn1* mutation was shown to decrease PP2A activity in vivo (Deruère et al., 1999). Therefore, these results indicate that the RCN1 PP2A acts as a positive regulator in ABA signaling in Arabidopsis guard cells.

### ABA Activation of Anion Channels Is Impaired in *rcn1* Guard Cells

The activation of slow (S-type) anion channels is important for ABA-induced stomatal closing (Schroeder and Hagiwara, 1989; Schroeder et al., 1993), and ABA has been shown to activate S-type anion channels in guard cells (Grabov et al., 1997; Pei et al., 1997, 1998; Leonhardt et al., 1999; Li et al., 2000). We tested the ABA activation of S-type anion channels in *rcn1* by preincubating guard cells in ABA and subsequently patch clamping the cells to assay for anion channel activities (Pei et al., 1997). The membrane potential was held at the anion equilibrium potential and stepped to membrane voltages ranging from −145 to +35 mV (Schroeder and Keller, 1992). As shown in Figures 2A and 2B, anion channel currents were activated by ABA in wild-type guard cells. By contrast, ABA failed to activate anion channels in *rcn1* guard cells (Figures 2C and 2D). These data suggest that RCN1 is a subunit of the OA-sensitive protein phosphatase that is responsible for the OA inhibition of ABA-activated anion channels. Impairment in the ABA activation of anion channels in *rcn1* is consistent with the ABA insensitivity of stomatal closing in *rcn1* guard cells (Figure 1C). The finding that short-term exposure of wild-type guard cells to OA (Pei et al., 1997) mimics the *rcn1* knockout phenotypes (partial inhibition of ABA-induced stomatal closing [Figure 1C] and anion channel activation [Figure 2]) suggests that ABA insensitivity in *rcn1* is not attributable to a long-term effect of the *rcn1* loss of function during guard cell development. Rather, these data suggest that RCN1 functions directly in ABA signaling.

### Mechanisms Downstream of Cytosolic $Ca^{2+}$ Show Wild-Type Responses in the *rcn1* Mutant

ABA-induced cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) increases occur upstream of anion channel activation (Schroeder and Hagiwara, 1989; McAinsh et al., 1990; Allen et al., 1999a). Extracellular $Ca^{2+}$ (McAinsh et al., 1995) and reactive oxygen species, a recently discovered second messenger of ABA signaling (Pei et al., 2000), induce increases in $[Ca^{2+}]_{cyt}$ that mediate stomatal closure. To determine whether the *rcn1* mutation can be bypassed by $[Ca^{2+}]_{cyt}$ increases, we performed stomatal closing assays with extracellular $Ca^{2+}$ and $H_2O_2$. As shown in Figure 3A, stomatal responses in the *rcn1* mutant showed no significant difference compared with those of the wild type when they were treated with 1 and 5 mM external $Ca^{2+}$ ($P > 0.32$ at 1 mM $Ca^{2+}$ and $P > 0.11$ at 5 mM $Ca^{2+}$). Furthermore, stomatal responses to 100 μM $H_2O_2$ were similar in *rcn1* and the wild type ($P > 0.54$; Figure 3B). These results suggest that mechanisms downstream of $[Ca^{2+}]_{cyt}$ increases, which lead to stomatal closure, may be largely functional in the *rcn1* mutant.

### ABA-Induced Cytosolic $Ca^{2+}$ Increases Are Reduced in *rcn1* Guard Cells

One of the earliest measurable ABA signaling events in guard cells is an ABA-induced increase in $[Ca^{2+}]_{cyt}$ (McAinsh et al., 1990, 1997; Schroeder and Hagiwara, 1990). Therefore, to determine whether the *rcn1* mutation affects mechanisms upstream of $[Ca^{2+}]_{cyt}$ increases in guard cells, we performed time-resolved $[Ca^{2+}]_{cyt}$ imaging studies using wild-type and *rcn1* plants expressing the $Ca^{2+}$ reporter yellow cameleon 2.1 (Miyawaki et al., 1997; Allen et al., 1999b). Two independent wild-type lines and three independent *rcn1* lines transformed with yellow cameleon 2.1 were used for $[Ca^{2+}]_{cyt}$ imaging experiments. We treated guard cells with 5 μM ABA to investigate the responsiveness of ABA-induced $[Ca^{2+}]_{cyt}$ increases in *rcn1* because guard cells of *rcn1* showed ABA insensitivity in stomatal movements (Figure 2B) and 5 μM ABA induced $[Ca^{2+}]_{cyt}$ increases in wild-type guard cells (Wassilewskija ecotype).

Figures 4A and 4B show representative ABA-induced $[Ca^{2+}]_{cyt}$ increases in wild-type and *rcn1* guard cells. Five micromolar ABA induced two or more calcium transients in 44% of wild-type guard cells ($n = 12$ of 27 cells; Figures 4A, top trace, and 4C). By contrast, only 14% of *rcn1* guard cells showed two or more calcium increases ($n = 5$ of 37 cells; Figures 4B, top trace, and 4C). Furthermore, in *rcn1*, 62% of guard cells showed no response to 5 μM ABA ($n = 23$ of 37 cells; Figures 4B, bottom trace, and 4C), whereas 37% of wild-type guard cells showed no response to 5 μM ABA during 40 to 45 min of recordings ($n = 10$ of 27 cells; Figures 4A, bottom trace, and 4C). Note that even at higher ABA concentrations, a background rate of ~25 to 30% of wild-type guard cells showed no measurable ABA-induced $[Ca^{2+}]_{cyt}$ increases under the imposed conditions (Hugouvieux et al., 2001). The number of cells that showed ABA-induced $[Ca^{2+}]_{cyt}$ increases, including single $Ca^{2+}$ transient responses, was reduced significantly in *rcn1* guard cells ($\chi^2 = 3.9$, $P < 0.03$). Blind analyses of the same and unmarked data sets confirmed the reduced ABA induction of $[Ca^{2+}]_{cyt}$ increases in *rcn1* guard cells (see Methods). The average ABA-induced $[Ca^{2+}]_{cyt}$ amplitude ratio changes were not significantly different between wild-type and *rcn1* guard cells (211.6 ± 95.2 nM for the wild type and 222.0 ± 68.2 nM for *rcn1*; $P > 0.71$). However, the number of ABA-induced $[Ca^{2+}]_{cyt}$ transients was reduced in *rcn1* guard cells compared with the wild type, even when nonresponsive cells (no transients) were excluded from the analyses (2.6 ± 1.7 per recording for the wild type and 1.6 ± 0.9 per recording for *rcn1*; $P < 0.05$). Calcium imaging data indicate that the *rcn1*

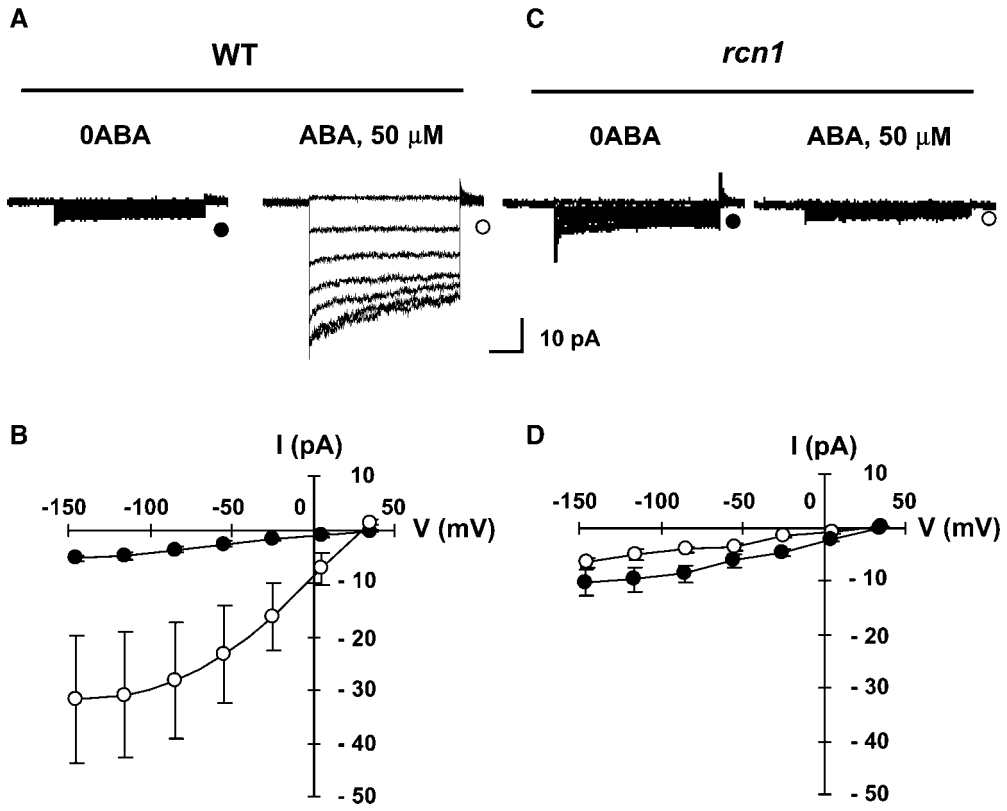


**Figure 2.** The *rcn1* Mutation Impairs the ABA Activation of S-Type Anion Channels in Guard Cells.

**(A)** Whole cell recordings of anion channel currents in wild-type (WT) guard cells in the absence (left trace) or presence (right trace) of 50 μM ABA.

**(B)** Average peak current-voltage relationships for ABA activation of anion currents in wild-type cells as recorded in **(A)** (closed circles, 0 ABA, $n = 8$; open circles, 50 μM ABA, $n = 10$). Error bars represent standard errors.

**(C)** Whole-cell recordings of anion channel currents in *rcn1* guard cells in the absence (left trace) or presence (right trace) of 50 μM ABA. ABA failed to activate anion channel currents in *rcn1*.

**(D)** Average peak current-voltage relationships for ABA activation of anion currents in *rcn1* cells as recorded in **(B)** (closed circles, no ABA, $n = 6$; open circles, 50 μM ABA, $n = 7$). Error bars represent standard errors.

mutation partially reduced the responsiveness of guard cells to ABA by reducing the probability of ABA-induced $[Ca^{2+}]_{cyt}$ increases.

We further tested whether the PP1/PP2A inhibitor OA applied to wild-type guard cells can mimic the *rcn1* phenotype for ABA-induced $[Ca^{2+}]_{cyt}$ increases. In the absence of OA, ABA triggered one or more calcium transients in 69% of guard cells (Wassilewskija ecotype) ($n = 20$ of 29 cells; 9 cells with one transient, 11 cells with two or more transients) (Figure 4D, WT − OA). However, in the presence of 1 μM OA, ABA triggered one or more calcium increases in only 23% of guard cells ($n = 7$ of 30 cells; four cells with one transient, three cells with two or more transients) (Figure 4D, WT + OA). Moreover, in the presence of 1 μM OA, 77% of guard cells showed no response to 5 μM ABA ($n = 23$ of 30 cells; Figure 4D); in the absence of OA, 31% of guard cells showed no response to 5 μM ABA ($n = 9$ of 29 cells; Figure 4D). These results show that 1 μM OA significantly reduced the number of guard cells displaying ABA-induced $[Ca^{2+}]_{cyt}$ increases ($\chi^2 = 12.27$, $P < 0.0005$), demonstrating that OA applied to the wild type phenocopies the *rcn1* phenotype.

### The *rcn1* Disruption Mutation Reduces ABA Sensitivity in Seeds

To examine the expression of *RCN1* in other plant tissues, we performed RNA gel blot analyses with poly($A^+$) mRNA extracted from Arabidopsis flowers, leaves, stems, and roots. As shown in Figure 5A, *RCN1* was expressed in all organs, suggesting additional roles for RCN1 (Corum et al., 1996; Garbers et al., 1996).

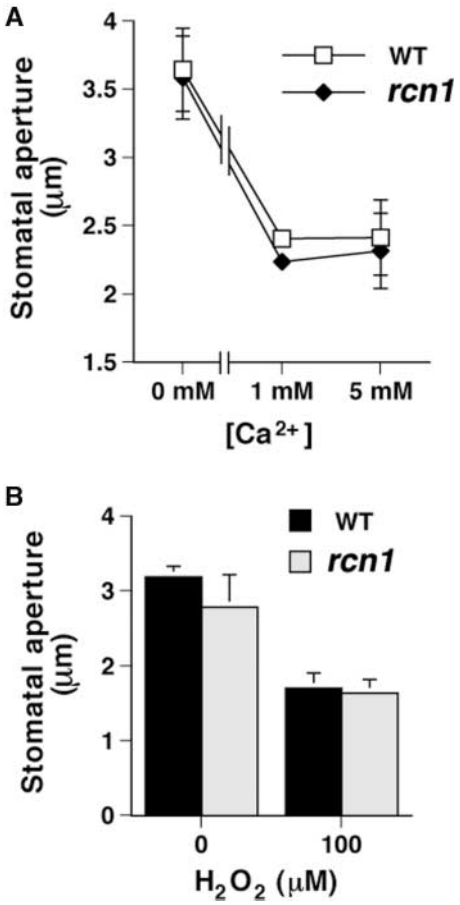


**Figure 3.** Calcium- and $H_2O_2$-Induced Stomatal Closing Movements Are Not Affected in *rcn1*.

**(A)** Stomatal apertures were measured 3 h after the addition of 0, 1, or 5 mM $Ca^{2+}$ to the solution of wild-type (WT) and *rcn1* stomata that had been preopened in white light. Error bars represent standard errors relative to three independent experiments with 60 stomata per data point.

**(B)** Stomatal apertures were measured 3 h after the addition of 100 μM $H_2O_2$ to the bath solution of wild-type and *rcn1* stomata that had been preopened in light for 3 h. Error bars represent standard errors relative to three independent experiments with 80 (wild type) and 60 (*rcn1*) stomata per data point. The light fluence rate was 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

ABA mediates dormancy by inhibiting seed germination. We examined whether PP1 or PP2A may function in ABA-mediated inhibition of seed germination. Wild-type seed germination rates were measured on plates containing 0, 0.5, 1, 2, or 5 μM ABA in the absence and presence of OA. Seed germination was analyzed 5 days after transfer to a growth chamber preceded by exposure to 4°C for 4 days. As shown in Figure 5B, ABA inhibited the germination of wild-type Arabidopsis seeds. Interestingly, ABA inhibition of wild-type seed germination was reduced dramatically in the presence of 1 μM OA ($P < 0.0005$ at 1 μM ABA, $P < 0.001$ at 2 μM ABA, and $P < 0.006$ at 5 μM ABA). OA applied to seeds in the absence of added ABA did not affect seed germination, resulting in 99.7% germination (Figure 5B, 0 ABA). These results provide evidence that PP1s or PP2As also act as positive transducers of ABA signaling in Arabidopsis seeds.

To determine whether the loss of function of *RCN1* affects the ABA regulation of seed germination in Arabidopsis, we investigated the effects of the *rcn1* mutation on seed germination. ABA inhibition of seed germination showed a partial ABA insensitivity in the *rcn1* mutant ($P < 0.008$ at 0.5 μM ABA, $P < 0.01$ at 1 μM ABA, and $P < 0.02$ at 2 μM ABA; Figure 5C). Two independent complemented lines of *rcn1* showed seed germination rates similar to those of wild-type lines (for *rcn1*g17-1, $P > 0.78$ at 0.5 μM ABA, $P > 0.88$ at 1 μM ABA, and $P > 0.20$ at 2 μM ABA; for *rcn1*g8-1, $P > 0.26$ at 0.5 μM ABA, $P > 0.05$ at 1 μM ABA, and $P > 0.58$ at 2 μM ABA; Figure 5C). These results show that RCN1 contributes to the ABA inhibition of seed germination in Arabidopsis as well as to ABA signaling in guard cells. The stronger effect of OA on the ABA regulation of seed germination (Figure 5B) compared with the *rcn1* phenotype (Figure 5C) indicates that additional (partially redundant) OA-sensitive protein phosphatases may function in ABA signaling in seeds.

### Reduced Expression of ABA-Responsive Genes in *rcn1*

To determine whether the *rcn1* mutation also alters the expression of ABA-responsive genes, RNA gel blot analyses were performed. Total RNA was extracted from rosette leaves of wild-type and mutant plants sprayed with water or ABA. We hybridized RNA gel blots with probes that have been used to examine ABA-inducible gene transcription in ABA signaling mutants (Xiong et al., 2001a, 2001b). The *rcn1* mutation reduced the ABA-induced transcript levels of *KIN2*, *KIN1* (Figure 6), and *RD29A* (data not shown). Consistent with these results, ABA-induced transcript levels of these genes were enhanced in the ABA-hypersensitive mutants *ade1* and *fry1-1* (Foster and Chua, 1999; Xiong et al., 2001b).

## DISCUSSION

The recessive T-DNA disruption mutant *rcn1* reduces the ABA responsiveness of Arabidopsis seed germination, ABA-induced stomatal closing, ABA activation of S-type anion channels, and ABA-induced gene expression. These findings suggest that RCN1 is a general component of ABA signaling. The *rcn1* mutation partially reduces sensitivities to ABA by decreasing the probability of ABA-induced $[Ca^{2+}]_{cyt}$ increases in guard cells (Figure 4), whereas experimental

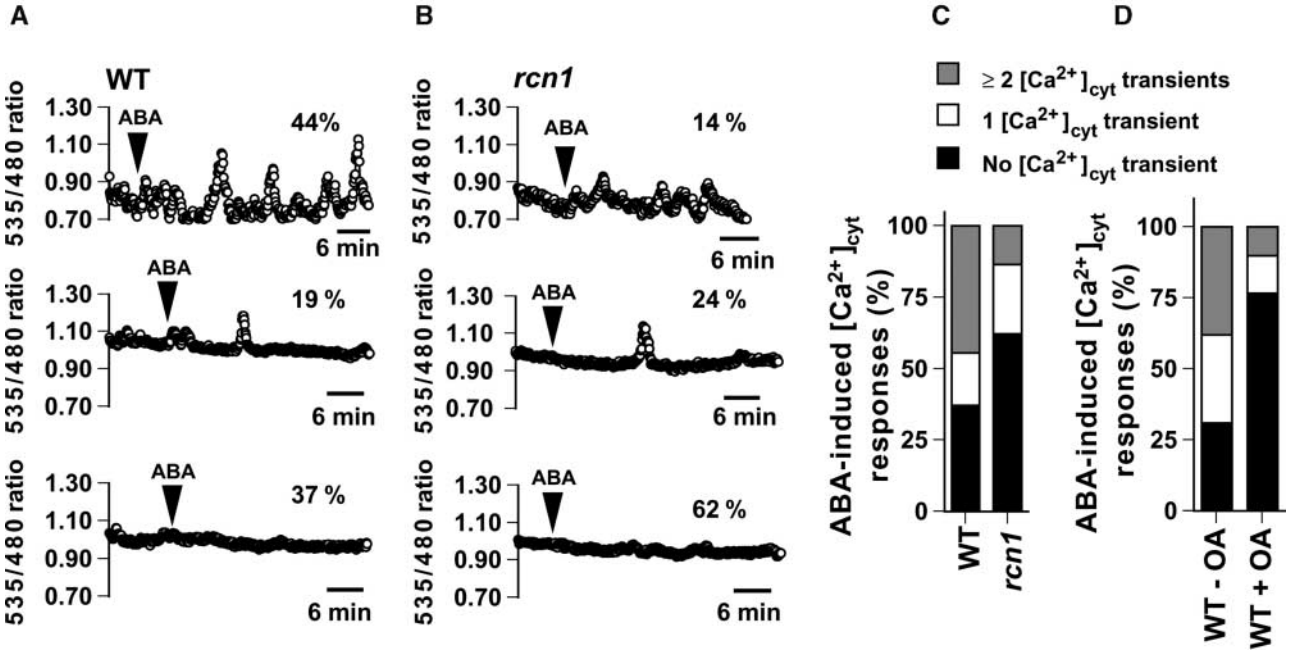


**Figure 4.** ABA-Induced $[Ca^{2+}]_{cyt}$ Increases Are Reduced in *rcn1* Guard Cells.

**(A)** The fluorescence emission ratio (535/480 nm) shows examples of ABA-induced $[Ca^{2+}]_{cyt}$ increases at 5 μM ABA in wild-type (WT) guard cells ($n = 27$ cells).

**(B)** The fluorescence emission ratio (535/480 nm) shows examples of ABA-induced $[Ca^{2+}]_{cyt}$ increases at 5 μM ABA in *rcn1* guard cells ($n = 37$ cells).

In **(A)** and **(B)**, traces demonstrating two or more ABA-induced $[Ca^{2+}]_{cyt}$ transients are shown at top, those demonstrating one $[Ca^{2+}]_{cyt}$ transient are shown in the middle, and those demonstrating no clear $[Ca^{2+}]_{cyt}$ transient are shown at bottom. Arrowheads indicate when cells were treated with 5 μM ABA. $[Ca^{2+}]_{cyt}$ transients were counted when changes in $[Ca^{2+}]_{cyt}$ ratios were ≥0.1 units.

**(C)** Stack column representation of the number of ABA-induced $[Ca^{2+}]_{cyt}$ transients recorded in wild-type ($n = 27$) and *rcn1* ($n = 37$) guard cells at 5 μM ABA.

**(D)** Stack column representation of the number of ABA-induced $[Ca^{2+}]_{cyt}$ transients recorded in wild-type guard cells at 5 μM ABA in the absence (WT − OA; $n = 29$ cells) or presence (WT + OA; $n = 30$ cells) of 1 μM OA.

$[Ca^{2+}]_{cyt}$ increases analyzed here bypass *rcn1*, resulting in stomatal closure in *rcn1* (Figure 3). The subtlety of the ABA response phenotype (30 to 40% higher germination frequencies at 1 μM ABA) could explain why mutations in the *RCN1* gene have not been isolated previously in forward genetic ABA response screens that set a higher threshold for facilitated mutant isolation.

### RCN1 Functions in ABA-Induced $[Ca^{2+}]_{cyt}$ Increases

In Arabidopsis suspension cells, recent studies have shown that ABA-induced $[Ca^{2+}]_{cyt}$ increases and anion channel activation are important for the ABA induction of *RAB18* gene expression (Ghelis et al., 2000a, 2000b). Furthermore, antisense suppression of the phospholipase C, *AtPLC1*, and sense expression of the inositol 1,4,5-trisphosphate 5-phosphatase, *AtIP5PII*, as well as the *fry1* mutation in an inositol polyphosphate 1-phosphatase implicate $Ca^{2+}$ signaling in the ABA inhibition of seedling emergence (Sanchez and Chua, 2001) and ABA-induced RD29A::luciferase expression (Xiong et al., 2001b). These findings suggest that cytosolic $Ca^{2+}$ may be a general second messenger for several ABA responses.

Calcium imaging experiments showed that the *rcn1* mutation impairs ABA-induced $[Ca^{2+}]_{cyt}$ increases in guard cells (Figure 4). Recent findings with experimentally imposed $Ca^{2+}$ oscillations in guard cells showed that the degree of stomatal closure depends on the number of $[Ca^{2+}]_{cyt}$ transients (Allen et al., 2001). The probability of ABA-induced $[Ca^{2+}]_{cyt}$ increases and the number of ABA-induced $[Ca^{2+}]_{cyt}$ transients were reduced significantly in *rcn1* guard cells. Stomatal movement experiments showed that experimental increases in $[Ca^{2+}]_{cyt}$ in *rcn1* can restore wild-type responses (Figure 3A).

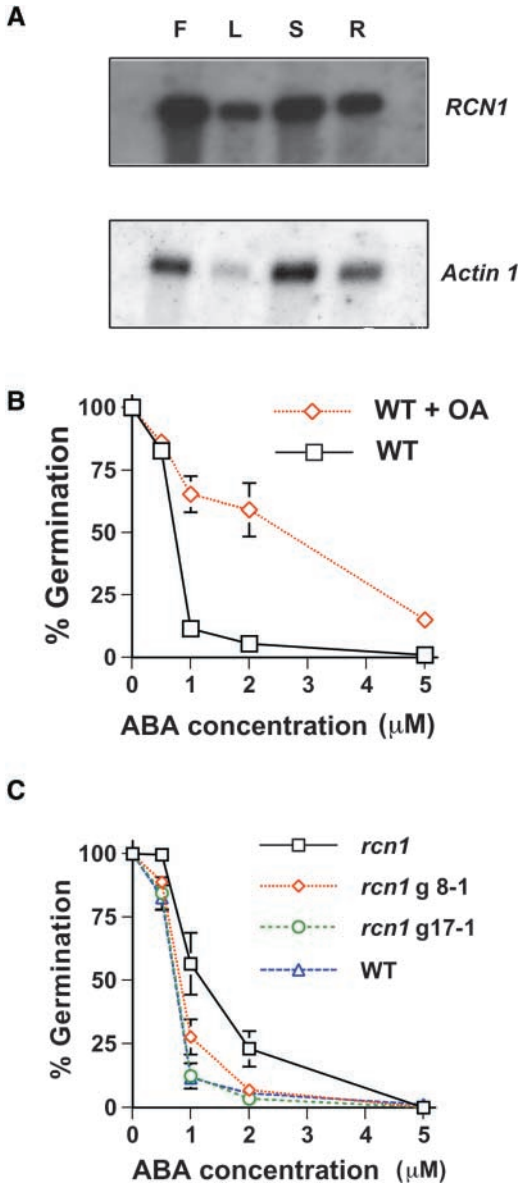


**Figure 5.** OA and the *rcn1* Mutation Both Impair ABA Inhibition of Arabidopsis Seed Germination.

**(A)** Tissue expression of the *RCN1* gene. *RCN1* transcript was detected in all organs examined (F, flower; L, leaf; S, stem; R, root). Two micrograms of poly(A$^+$) RNA was transferred onto a nylon membrane. The blot was hybridized with $^{32}$P-labeled *RCN1* and *Actin1* cDNA.
**(B)** ABA inhibition of wild-type (WT) seed germination is reduced in the presence of 1 μM of OA. Error bars represent standard errors of three independent experiments with >360 seeds at each data point.
**(C)** The *rcn1* mutation causes partial reduction in ABA sensitivity of seed germination. Wild-type seed germination rates were observed in two independent *rcn1*-complemented lines (*rcn1*g17-1 and *rcn1*g8-1). Symbols of the complemented *rcn1*g17-1 and *rcn1*g8-1 lines are overlapped with wild-type symbols when not visible. Error bars represent standard errors of three independent experiments with >360 seeds at each data point.

ABA is known to induce stomatal closing via multiple pathways (Allan et al., 1994; Grabov et al., 1997; Allen et al., 1999a; Pei et al., 2000). A previous study with the *abi1-1* and *abi2-1* PP2C mutants demonstrated that the addition of external calcium results in stomatal closing in both the wild type and PP2C mutants by activating processes downstream of $[Ca^{2+}]_{cyt}$ increases (Allen et al., 1999a). These data showed that *abi1-1* and *abi2-1* can be bypassed in the stomatal closing pathway. Furthermore, because *abi1-1* and *abi2-1* disrupt the ABA activation of plasma membrane $Ca^{2+}$ channels, the ability to impose $[Ca^{2+}]_{cyt}$ increases by adding external $Ca^{2+}$ suggests that the external $Ca^{2+}$-induced $Ca^{2+}$ oscillation pathway differs from the ABA-induced $Ca^{2+}$ oscillation pathway (Allen et al., 1999a; Murata et al., 2001). This hypothesis is strengthened by the finding that the *det3* mutant affects the external $Ca^{2+}$ pathway but not the ABA signaling pathway (Allen et al., 2000). Based on these previous findings, stomatal movement assays (Figure 3A) suggest that the impairment in ABA-induced stomatal closing in the *rcn1* mutant is bypassed by external calcium.

One micromolar OA phenocopies the *rcn1* phenotype in wild-type guard cells with ABA-induced $[Ca^{2+}]_{cyt}$ increases (Figure 4D), which correlates with findings that *rcn1* has reduced PP2A activity (Deruère et al., 1999). Together with reactive oxygen species–induced stomatal closing assays (Figure 3B), these data indicate that RCN1 may act upstream of reactive oxygen species and/or in a parallel branch of the ABA signaling network in guard cells (Blatt, 2000; Schroeder et al., 2001). The location of RCN1 relative to ABA-induced $Ca^{2+}$ release mechanisms (Leckie et al., 1998; Staxen et al., 1999) and ABA-activated plasma membrane $Ca^{2+}$ channels (Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001) remains to be determined.

## Cross-Talk in RCN1-Mediated Signaling

Recent studies have demonstrated that a single signal transduction protein can mediate cross-talk between two or more different hormone response pathways (Alonso et al., 1999; Ephritikhine et al., 1999; Beaudoin et al., 2000; Ghassemian et al., 2000; Lu and Fedoroff, 2000). For example, the Arabidopsis *sax1* mutant showed altered sensitivities to ABA, auxin, and gibberellins, but normal responses to these hormones were restored by exogenous application of brassinosteroid (Ephritikhine et al., 1999). The *rcn1* mutant was shown previously to be sensitive to the auxin transport inhibitor naphthylphthalamic acid in Arabidopsis (Garbers et al., 1996). The *rcn1* mutant exhibits defects in auxin transport, which may cause abnormal auxin distribution (Rashotte et al., 2001). Our analyses of early signaling events show that RCN1 functions in the early ABA signal transduction cascade. Our data provide additional evidence that a single molecular component can function in both auxin transport and ABA hormone response pathways.

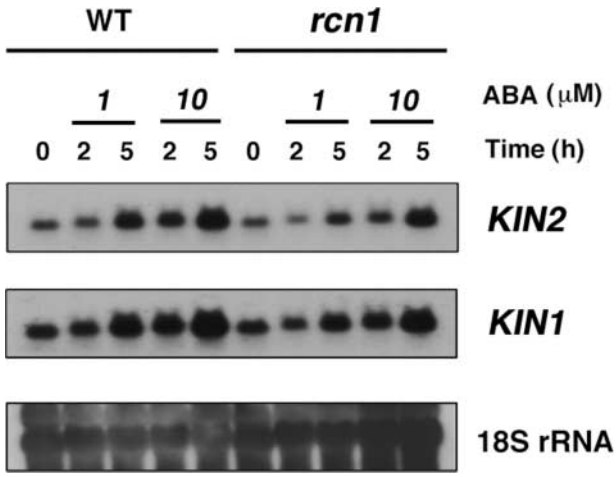


**Figure 6.** Transcript Levels of ABA-Responsive Genes Are Reduced in the *rcn1* Mutant.

Wild-type (WT) and *rcn1* plants were sprayed with 1 or 10 μM ABA 2 or 5 h before RNA isolation. Twenty micrograms of total RNA extracted from rosette leaves was transferred onto a nylon membrane. The blot was hybridized with $^{32}$P-labeled *KIN2* or *KIN1* cDNA. The same blot was hybridized with $^{32}$P-labeled 18S rDNA to show relative amounts of RNA samples.

PP2As are heterotrimeric holoenzymes that constitute a family of Ser/Thr phosphatases. A catalytic C subunit and a regulatory A subunit make up the heterodimeric AC core enzyme, which is associated with another regulatory B-type subunit (Janssens and Goris, 2001). In Arabidopsis, genes encoding three A subunits, five C subunits, two B subunits, eight B′ subunits, and six B′′ subunits have been identified. Given the numbers of PP2A subunit genes, permutations in the specific PP2A holoenzyme composition could provide diversity in PP2A activity (Janssens and Goris, 2001). In Arabidopsis, the yeast two-hybrid system and in vitro binding assays revealed that one A subunit can interact with two B′ subunits and two C subunits (Haynes et al., 1999), suggesting many possibilities in PP2A holoenzyme composition. Therefore, it is possible that the RCN1 PP2A subunit forms complexes with more than one PP2A catalytic subunit in vivo, which could contribute to cross-talk among RCN1 functions.

All five C subunit genes are expressed in seedlings and all organs of Arabidopsis (Ariño et al., 1993; Pérez-Callejón et al., 1993; Casamayor et al., 1994). RNA gel blot analyses show that three A subunit, two B subunit, one B′′ subunit, and five B′ subunit genes also are expressed ubiquitously (Rundle et al., 1995; Corum et al., 1996; Latorre et al., 1997; Haynes et al., 1999; Camilleri et al., 2002; Terol et al., 2002). However, GUS reporter gene fusion experiments showed that the promoters of one C and two B subunit genes have some tissue specificity in seedlings and flowers (Thakore et al., 1999). In addition, whole-mount in situ hybridization and promoter-GUS reporter fusions showed some tissue specificity of *RCN1* in roots, shoots, and etiolated hypocotyls (Deruère et al., 1999).

### RCN1 as a Positive Transducer of ABA Signaling

Reversible phosphorylation events play important roles in ABA signal transduction. Genetic screens have yielded two genetically dominant ABA-insensitive Arabidopsis mutants, *abi1-1* and *abi2-1* (Koornneef et al., 1984), in which two genes encoding PP2C have a point mutation (Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998). The mutant alleles of these genes are dominant, which supports the proposed redundancy of PP2Cs (Leung et al., 1997; Rodriguez et al., 1998). It is possible that the wild-type ABI1 and ABI2 proteins have additional functions. Because intragenic suppressors of the *abi1-1* and *abi2-1* mutants showed reduced or no protein phosphatase activity in vitro (Gosti et al., 1999; Merlot et al., 2001) and recessive ABA hypersensitivity was reported in a double mutant of both suppressors (Merlot et al., 2001), these PP2Cs have been proposed to act as negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001). Furthermore, overexpression of the constitutively active ABI1 and AtPP2C mutant isoforms in maize mesophyll protoplasts inhibited ABA-induced gene expression, also suggesting that PP2Cs are negative regulators of ABA signaling (Sheen, 1998). All suppressor mutants in *abi1-1* and *abi2-1* have point mutations downstream of the dominant G180D (*abi1-1*) and G168D (*abi2-1*) sites, and no stop codon or frameshift alleles have been isolated. Research with gene disruption/silencing mutants should allow further analysis of the model that the wild-type ABAI1 and ABI2 PP2Cs function as negative regulators of ABA signaling (Sheen, 1998; Merlot et al., 2001).

The present study demonstrates that in addition to PP2Cs, PP2As function in early ABA signal transduction. *rcn1* used in our study is a recessive ABA-insensitive gene disruption mutant that is complemented by the wild-type *RCN1* gene. A previous biochemical and pharmacological study showed that wild-type RCN1 protein increases PP2A activity in vivo and that *rcn1* shows reduced PP2A activity (Deruère et al., 1999). ABA responses in the wild type were inhibited by short-term OA exposure (Figures 4D and 5B) (Pei et al., 1997), which correlates with *rcn1* mutant phenotypes in ABA inhibition of seed germination, ABA-induced $[Ca^{2+}]_{cyt}$ increases, ABA-induced stomatal closing, and ABA activation of anion channels in Arabidopsis guard cells (Figures 1B and 2). These data provide evidence that RCN1 is a positive transducer of ABA signal transduction.

Pharmacological research with guard cells from other species and with other cell types has suggested that other PP1s or PP2As may function as negative regulators of ABA signal transduction (Schmidt et al., 1995; Esser et al., 1997;

Grabov et al., 1997; Wu et al., 1997). Other yet-to-be-identified PP1 or PP2A genes or protein complexes may act as negative regulators of ABA signaling. Our genetic and cell biological analyses have allowed a distinction between pharmacologically proposed PP1s and PP2As by showing that the RCN1 PP2A positively mediates ABA signal transduction.

## Conclusions

In conclusion, we have identified a guard cell–expressed PP2A regulatory A subunit gene, *RCN1*, which functions in ABA signal transduction. The *rcn1* loss-of-function mutation confers general ABA insensitivity to Arabidopsis and impairs ABA-induced $[Ca^{2+}]_{cyt}$ increases in guard cells, ABA activation of anion channels, and ABA-induced stomatal closing. Short-term exposure to the PP2A inhibitor OA in wild-type Arabidopsis phenocopies *rcn1*, further suggesting that RCN1 functions in ABA signal transduction. Furthermore, ABA inhibition of seed germination and ABA induction of gene expression are reduced in *rcn1*, suggesting that RCN1 is a general transducer of ABA signaling.

## METHODS

### Identification of the *RCN1* Gene in Guard Cells

To identify guard cell–expressed type 2A protein phosphatase (PP2A) genes, catalytic and regulatory subunit sequences of PP2A genes were aligned and then two conserved regions of PP2A regulatory A subunits were selected: AH/YVLLPPLE (for the sense primer) and DVRY/FFANQA (for the antisense primer). The degenerate oligomers designed from these sequences were 5′-GCIYAYGTIYTIYTICCICCIYTIGA-3′ (sense primer) and 5′-GCYTGRTTIGCRAARTAICKIACRTC-3′ (antisense primer). Total RNA was extracted from guard cell–enriched epidermal strips as described (Hugouvieux et al., 2001). Guard cell cDNA libraries were synthesized from guard cell total RNA (1 to ~2 μg) using the First-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). PCR was performed in a 50-μL mixture (sense and antisense primers at 200 nM, 1 × ExTaq polymerase buffer [Takara, Otsu, Japan], each deoxynucleotide triphosphate at 200 μM, 5 ng of cDNA, and 2.5 units of ExTaq polymerase). The PCR mixture was denatured at 94°C for 4 min followed by 35 cycles of amplification (94°C for 30 s, 45°C for 30 s, and 72°C for 3 min). PCR products were purified and cloned into the pGEM-T Easy vector (Promega). Sequencing of inserts was performed as described (Kwak et al., 1997).

### Stomatal Aperture Measurements

Leaves of 5- to 6-week-old wild-type, *rcn1*, and *rcn1*-complemented *Arabidopsis thaliana* plants (T4 progeny of *rcn1*g17-1 and *rcn1*g8-1) (Garbers et al., 1996) were incubated in white light (fluence rate of 125 or 83 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 2.5 h in stomatal opening solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM Mes/Tris, pH 6.15, except for the experiment illustrated in Figure 1C, in which 30 mM KCl, 50 μM $CaCl_2$, and 10 mM Mes/Tris, pH 6.15, was used as described by Pei et al. (1997). In the present study, stomatal apertures were measured in the focal plane of the outer edges of guard cells in epidermal strips. For calcium-induced stomatal closing experiments, the same opening solution without any calcium was used. Stomatal apertures were measured 3 h after abscisic acid (ABA), calcium, or $H_2O_2$ was added. Standard errors were calculated relative to the square root of the number of stomatal aperture experiments. Statistical significance was determined using Student's *t* test (two-tailed distribution, two samples assuming equal variance) and Excel software (version 98; Microsoft, Redmond, WA).

### Reverse Transcription PCR and β-Glucuronidase Activity Analyses

Leaves of 5- to 6-week-old wild-type Arabidopsis plants were used to prepare guard cell and mesophyll cell protoplasts as described (Leonhardt et al., 1997). Protoplast preparations with high purity (>97% for guard cells and >95% for mesophyll cells) were used to extract total RNA using Trizol reagent (Life Technologies, Rockville, MD). First-strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). The PCR mixture was prepared as described above and denatured at 94°C for 4 min followed by 27 cycles of amplification (94°C for 30 s, 57°C for 30 s, and 72°C for 2 min). Primers used for PCR were as follows: Actin1-5, 5′-GGCCGATGGTGAGGATATTCAGCCACTTG-3′; Actin1-3, 5′-TCGATGGACCTGACTCATCGTACTCACTC-3′; RCN1-51, 5′-CCGACGCCTGGATCGTGATTTGATTCGA-3′; and RCN1-31, 5′-CAATTCAGGATTGTGCTGCTGTGGAACCA-3′. β-Glucuronidase activity was assayed on 12-day-old wild-type transgenic seedlings grown on Murashige and Skoog (1962) plates as described (Hugouvieux et al., 2001).

### Patch-Clamp Analyses

Arabidopsis guard cell protoplasts were isolated enzymatically from leaves of 5- to 6-week old wild-type and *rcn1* plants as described previously (Kwak et al., 2001). Whole-cell recordings of guard cells were conducted as described (Pei et al., 1997). The pipette solution contained 150 mM CsCl, 5.87 mM $CaCl_2$, 2 mM $MgCl_2$, 6.7 mM EGTA, 5 mM Mg-ATP, and 10 mM Hepes/Tris, pH 7.1. The bath solution contained 30 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Mes/Tris, pH 5.6. Osmolalities were adjusted to 500 mmol/kg for the pipette solution and to 485 mmol/kg for the bath solution. Seal resistance was >10 GΩ. The holding potential was +30 mV. Subsequent voltage steps were decreased by 30 mV per pulse. To measure the effects of the upstream ABA signaling pathway on slow anion channel activities, protoplasts were preincubated at 22°C with 50 μM ABA for 2 h before patch-clamp recordings (Pei et al., 1997). Leak currents were not subtracted.

### Calcium Imaging Analyses

Four- to 6-week old wild-type (two independent lines) and *rcn1* (three independent lines) plants stably transformed with the yellow cameleon construct p35SYC2.1 were used for calcium imaging analyses as described (Allen et al., 1999b; Hugouvieux et al., 2001). Epidermal strips were incubated in white light (fluence rate of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 2.5 h in stomatal opening solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM Mes/Tris, pH 6.15, before recordings. To

test okadaic acid (OA) effects on ABA-induced cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) increases in wild-type guard cells, epidermal strips were incubated in the dark for 2.5 h in the same solution in the absence or presence of 1 μM OA before recordings. Background fluorescence was measured in guard cell-less epidermal domains (~20% of cameleon fluorescence intensity) and was subtracted from the epidermal field before ABA application. Chloroplast fluorescence in guard cells was not observed because of changes in the optical setup and the use of higher-cameleon-expressing plants (Allen et al., 1999b). Statistical analyses demonstrated that the average fluorescence baseline ratio before ABA application was not significantly different in wild-type (0.87 ± 0.15) and *rcn1* (0.83 ± 0.14; $P > 0.12$) plants. $[Ca^{2+}]_{cyt}$ transients were counted when the change in $[Ca^{2+}]_{cyt}$ ratios was ≥0.1 units above the baseline. More than 50% of cells showed spontaneous $Ca^{2+}$ transients and were excluded from analyses (Allen et al., 1999b). Cells showing stable $[Ca^{2+}]_{cyt}$ ratios during the first 7 min were exposed to ABA. Unmarked and mixed complete data sets were analyzed as blind tests in which the identities of the plant lines (wild-type or *rcn1*) under investigation were not known to two independent researchers, confirming the reduced ABA responsiveness in *rcn1* (V. Hugouvieux, $\chi^2 = 3.9$, $P < 0.03$; J. Young, $\chi^2 = 4.9$, $P < 0.03$).

### Seed Germination Analyses

Seeds of wild-type, *rcn1*, and two independent *rcn1*-complemented lines were plated on one-fourth-strength Murashige and Skoog (1962) medium containing 0, 0.5, 1, 2, or 5 μM ABA. Seeds were stratified at 4°C for 4 days and then transferred to a growth chamber (24°C under a 16-h-light/8-h-dark regime). Seed germination rates were scored after 5 days in the growth chamber. To test the effect of OA, 1 μM OA was added to medium containing the indicated ABA concentrations.

### RNA Gel Blot Analyses

Total RNA was extracted from flowers, leaves, stems, and roots of 5- to 6-week-old wild-type plants as described above. Poly($A^+$) RNA was further purified using the μMACS mRNA Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. To determine ABA-inducible gene expression, total RNA was extracted from rosette leaves of wild-type and *rcn1* plants sprayed with 1 or 10 μM ABA for 2 and 5 h. Total and poly($A^+$) RNA were separated on a 1.2% (w/v) denaturing agarose gel and then transferred onto a Hybond-$N^+$ membrane (Amersham Pharmacia Biotech). The blots were hybridized with $^{32}$P-labeled *RCN1*, *KIN1*, or *KIN2* cDNA. Blots were washed as described (Kwak et al., 1997). $^{32}$P-labeled *Actin1* cDNA or 18S rDNA was used to show relative amounts of RNA samples.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

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